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Research Article

Impact of an algae feed supplement administration on the carriage of virulent *Escherichia coli* ESBL producing in post-weaned piglets

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This study aimed to determine the impact of a feed supplement administration on *E. coli* virulent strains in piglets. A breeding of four batches of two piglets was carried out. Batch A served as controls and batches B, C and D were administered respectively with Algo-Bio®, tetracycline and penicillin-streptomycin combination therapy. *E. coli* strains were isolated from faeces on Mac Conkey medium, and identify them. Antibiotic susceptibility test was performed using disk diffusion method on Müller-Hinton agar, and then virulence genes were determined by PCR. Antibiotic susceptibility test revealed 58 *E.coli* ESBL-producing strains. Serotyping of these strains showed O128 (36.2%), O111 (20.6%), O119 (18.9%), O124 (15.5%) and O125 (8.6%). The prevalence of strains harboring *eaeA*, *Stx2*, *st* and *lt* genes increased in control piglets from D0 to D4. The strains prevalence hosting *eaeA*, *st* and *lt* genes increased from 50%, 75% and 100% respectively on D0 to 0 on D4. The percentages of strains harboring the *eaeA*, *Stx2* and *lt* genes decreased from 75% to 46.6%, from 50% to 40%, from 75% to 53.3% respectively in piglets treated with tetracycline. Algo-Bio® administration decreased virulent strains in piglet microbiota. This supplement could promote better control of antibiotic resistance risks in pig farming.

Key words: *Escherichia coli*, Piglets, Resistance, Virulence gene, Algo-Bio®

INTRODUCTION

Weaning is a health-critical phase in pig farming, where animals are subjected to a series of disturbances that weaken their digestive health. This makes them susceptible to digestive diseases in general, but particularly to diarrhea of infectious origin (Akujobi et al., 2008). In fact, piglets become very sensitive to digestive pathologies because of the fragile balance between the so-called beneficial flora and pathogenic bacteria after withdrawal of breast milk (Berri et al., 2015). Among the bacteria responsible for these pathologies, one example is *Escherichia coli*, responsible for post-weaning colibacillosis in piglets (Brooks et al., 2005). These bacteria belong to Enterobacteriaceae family and their ecological niche is the intestinal tract of animals and humans (CASFM, 2017; Corrégé. 2013). Several *Escherichia coli* pathovars (EPEC) can acquire genetic elements enabling them to produce virulence factors, to adapt to new niches and thus cause diarrhea. Post-weaning colibacillosis is a disease that manifests itself as diarrhea usually occurring a few days

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after weaning. It leads to rapid dehydration and mortality. This transmissible disease is caused by strains of *Escherichia coli* belonging to the class ETEC (Enterotoxigenic *Escherichia coli*) (Dadié et al., 2000). Colibacillosis is found in all types of animal husbandry, including high health status farms. This pathology represents the first cause of treatment and preventive use of antibiotics in animal husbandry, leading to the emergence of enterobacteria resistant to antibiotics most commonly used (Dadié et al., 2010). Indeed, antibiotic therapy promotes the pressure to select multi-resistant strains. These virulent and resistant enterobacteria can be transmitted to humans through direct contact with animals. They can also be transmitted through consumption of products from infected animals or through products from market gardening (manure application to crops). In addition, they can transmit their resistance and virulence genes to commensal and infectious bacteria of human origin and lead to public health problems (Akujobi et al., 2008; Fairbrother et al., 2006). On the other hand, consumers demand natural products of good microbiological quality and producers must always maintain high quality standards for their products. It is therefore become necessary to develop new natural alternatives solution to antibiotic therapy that promotes the health and growth of piglets. The objective of this work was to evaluate the impact of an algae-based feed supplement (Algo-Bio®) on the carriage of virulent strains of *Escherichia coli* producing ESBL in piglets, compared to tetracycline and penicillin-streptomycin combination therapy commonly used in Côte d'Ivoire in pig farming.

MATERIALS AND METHODS

Samples

Eight recently weaned and untreated piglets divided into 4 batches (A, B, C and D) were used. Piglets from batch A served as controls, piglets from batch B and C were administered with an algae-based food supplement (Algo-Bio®) and tetracycline respectively, orally in drinking water for 5 days and batch D were administered with penicillin-streptomycin combination therapy intramuscularly for 5 days too. Then fresh faeces of piglets from all batches were collected every morning (corresponding to D0, D1, D2, D3, D4) in well labeled sterile coproculture pots and transported to laboratory in a cooler with ice packs. Once at lab, the samples were analyzed within the hour. A total of forty stool samples were taken.

Isolation of *Escherichia coli* strains from faeces

For the isolation of *Escherichia coli* strains, 5 g of faeces were collected and homogenized in 45 ml of buffered peptone water. Decimal dilutions were made from the stock suspension, then 100 µl of these different dilutions were inoculated by spreading on Mac Conkey medium (Bio-rad, France) prepared according to manufacturer's specifications and cast in Petri dishes. The various cultures were incubated at 37°C for 24 hours. For each sample, small pink to pinkish-red colonies with or without haloes, suspected of *Escherichia coli* were collected and a subculture was streaked on Rapid *E. coli* (Bio-rad, France). Cultures were incubated at 37°C for 24 hours. The characteristic colonies of *Escherichia coli*, ranging in color from purple to pink, were selected for confirmation with the tests to identify the morphological and biochemical characteristics of this bacterium.

Determination of ESBL-producing strains of *Escherichia coli*

Antibiotic susceptibility test was performed on 641 strains of *Escherichia coli* by the Müller-Hinton (Bio-rad, France) agar gel diffusion method according to recommendations of Antibiogram Committee of the French Society of Microbiology (CASFM, 2017). Production of extended-spectrum beta-lactamase was confirmed by a synergistic assay in which the clavulanic acid-associated amoxicillin disc, aztreonam disc and third-generation cephalosporin discs were added to amoxicillin disc with clavulanic acid at distance of 2 cm center-to-center. Antibiotics (Bio-rad, France) tested were: amoxicillin/clavulanic acid (20 µg); cefepime (30 µg); cefotaxime (5 µg); ceftazidime (10 µg); aztreonam (30 µg); tetracycline (30 µg); amoxicillin (20 µg); chloramphenicol (30 µg); trimethoprim-sulfamethoxazole (1.25-23.75 µg); colistin (50 µg); imipenem (10 µg); ciprofloxacin (5 µg); piperacillin (30 µg); nalidixic acid (30 µg).

Serotyping of *Escherichia coli* strains

Serogrouping was carried out on ESBL-producing strains of *Escherichia coli* and was directed to serotypes with zoonotic potential, namely enteropathogenic strains. EPEC and O157, increasingly implicated in human cases of hemorrhagic colitis (Fleury., 2015). This guidance was based on frequency of association with human infections and their implications in epidemics. To carry out serogrouping, a colony portion of an 18-hour culture of *Escherichia coli* at 37°C on non-selective agar was suspended on a clean slide in a drop of physiological water (NaCl 9%). In agglutination presence, it is a strain with self-agglutination properties and therefore cannot be serotyped. In absence of agglutination, the assay was performed by splashing a portion of the strain in a drop of agglutinating serum containing specific antibodies (Bio-rad, France). A positive reaction resulted in agglutination which determined the serotype of strain.

Determination of virulence genes by PCR

DNA extraction of these bacteria and control strains was carried out on 24 hours cultures. The heat shock method was used. Genetic profiles of *Escherichia coli* virulence strains were obtained from simplex amplification reactions in reaction mixtures of 25 µL containing 11.8 µL water for injection, 2.5 µL colored buffer (5x Green GoTaq®), 2.5 µL of unstained buffer (5x Colorless GoTaq®), 1.5 µL of MgCl2 at 25 mM, 0.5 µL of dNTP at 10 mM, 1.5 µL of Forward primer, 1.5 µL of Reverse 20 mM primer, 0.2 µL of DNA polymerase (GoTaq®, Promega, USA) and 3 µL of extracted DNA. Primers used to search for strain virulence genes are listed in Table 1. Reaction mixtures without DNA were considered negative controls. *E. coli* M19 (Gordon et al., 2003; Hémonic et al., 2014) were used as positive control for *eaeA* and *stx2* genes, *E. coli* ATCC 35401 (Hémonic et al., 2013) were used as positive control for *st* and *lt* genes. Then, the individual reaction mixtures were placed in the wells of the thermal cycler plate (GeneAmp PCR System 9700, Applied Biosystems) for amplification. Amplification conditions for gene detection are listed in Table 2. Separation of the various amplification products was performed by 1.5% agarose gel electrophoresis.

Table 1. Acute toxicity after oral and parenteral administration.

Gene	Primer	Sequence (5'->3')	PCR product size (pb)	References
eaeA	*eae-F*	CACACGAATAAACTGACTAAAATG	367	(Kouamé et al.,
	eae-R	AAAAACGCTGACCCGCACCTAAAT		2017)
Stx2	*Stx2-F*	ACCGTTTTTCAGATTTTACACATA	298	(Dadié et al.,
	Stx2-R	TACACAGGAGCAGTTTCAGACAGT		2014)
st	*st-F*	TTTCCCCTCTTTTAGTCAGTCAACTG	160	(Dadié et al.,
	st-R	GGCAGGATTACAACAAAGTTCACAG		2014)
lt	*lt-F*	TCTCTATGTGCATACGGAGC	322	(Dadié et al.,
	lt-R	CCATACTGATTGCCGCAAT		2014)

Table 2. Amplification conditions for virulence genes.

Steps	Amplification condition/duration	
	Stx2	***eae, st, lt***
Initial denaturation	94°C/3 min	94°C/3 min
Cyclic denaturation	94°C/30 s	94°C/30 s
Hybridization	57°C/45 s	56°C/20 s
Cyclic elongation	72°C/30 s	72°C/30 s
Final elongation	72°C/5 min	72°C/5 min
Cycles	35	35

Dietary supplement efficacy test on virulent strains of *Escherichia coli* in vitro

Before carrying out in vitro efficacy tests of Algo-Bio®, a sterility test was carried out on the food supplement in order to verify that the substance is germ-free. Virulent *Escherichia coli* isolated in this study were used for in vitro efficacy testing. A total of four (4) virulent strains of *Escherichia coli*, and one (1) *E.coli* reference strain ATCC 25922 were used. These strains were streaked on regular agar. After 24 h incubation at 37°C, one colony of each strain was collected and placed in a tube containing 2 mL of physiological water (NaCl 85%) and homogenized. The density of the mixture was adjusted to 0.5 McFarland. Concentrations of 100%, 50% and 25% of the Algo-Bio® were prepared in sterile distilled water. They were introduced into wells made on MH agar, previously inoculated by swabbing with inoculum. A solution of equal volume of sterile water was introduced into another well and served as negative control. Antibiotic discs (amoxicillin, cefotaxime, cefepime, tetracycline) were used as positive controls. Then, the Petri dishes were incubated at 37°C for 24 hours.

The diameter of inhibition discs around each cup was measured using a caliper. The assessment of the efficacy of extracts was made according to the criterion of (Koné et al., 2019). Thus, a substance is said to be ineffective if the inhibition diameter is less than 8 mm, whereas it is said to be effective if the diameter is between 9 and 14 mm. On the other hand, it is considered very effective if the diameter is between 15 and 19 mm and extremely effective if the diameter is greater than 20 mm.

Statistical analysis

The statistical analysis of the data has been performed with XLSTAT version 2017. Statistical comparisons of virulence genes proportions in piglet groups according to different types of treatment were made with non-parametric Chi-square test. Statistical differences with a probability value of less than 5% ($p<0.05$) were considered significant. When the probability is greater than 5% ($p>0.05$) the statistical differences are not significant.

RESULTS

Prevalence of *Escherichia coli* strain serotypes

Out of the 641 *Escherichia coli* strains isolated from piglets and subjected to antibiotic susceptibility testing, 330 were found to be resistant and 58 produced ESBL. i.e. 51.4% resistant and 17.5% producing ESBL. *Escherichia coli* producing Extended Spectrum Beta-Lactamase (n=58) were selected for serotyping. Most of the strains of serotypes O128 were observed (21 strains) while only 5 strains of serotypes O125 were counted. The results of serotyping are shown in Figure 1.

Electrophoretic profiles of *Escherichia coli* virulence genes

PCR amplification using specific primers that code for strain virulence and amplicons separation by agarose gel electrophoresis showed specific bands for genes researched. Figure 2 shows electrophoretic profile of *eaeA* virulence genes (367 bp) of *Escherichia coli* strains. Electrophoretic profile of amplification product of *lt* virulence gene is shown in Figure 3. The gene size is 322 bp. The electrophoretic profile of amplification product of *st* virulence gene is shown in Figure 4. The gene size is 160 bp. Electrophoretic profile of amplification product of *Stx2* virulence gene is shown in Figure 5. The gene size is 298 bp.

Prevalence of virulence genes of *E. coli* strains before and after treatment

Proportions of strains hosting *E. coli* virulence genes

before the start of treatment and at the end of treatment are shown in Table 3. These high rates in piglets treated with Algo-Bio® before the start of treatment (D0) decrease significantly at the end of treatment (D4). However, in control piglets, strains frequencies increased in all observed virulence genes. In piglets treated with tetracycline, the proportions of strains decrease significantly but remain relatively high at the end of treatment (D4). In piglets treated with the penicillin-streptomycin combination, the proportions of strains hosting *eaeA* and *lt* genes decreased from 50% to 33.3% and 25% to 0 respectively while the proportions of strains hosting *st* genes increased from 50% to 66.6%. Chi-square test data showed a significant increase ($p<0.05$) in strains harboring the *eaeA*, *st* and *lt* genes in these piglets between the start of treatments (D0) and the last day of treatment (D4).

Sensitivity of *Escherichia coli* virulent to Algo-Bio® in solid media

All *Escherichia coli* tested showed sensitivity to Algo-Bio® concentrations C1 (100%) and C2 (50%). At C3 (25%), only the strains *Escherichia coli* EC72 showed no sensitivity with inhibition zone diameters of 6 mm. The best activity with the different concentrations C1, C2 and C3 of Algo-Bio® was observed with *Escherichia coli* EC05. The diameters of the inhibition zones observed with this strain were 26.5 mm, 21.5 mm and 19.5 mm at C1, C2 and C3 respectively (Table 4).

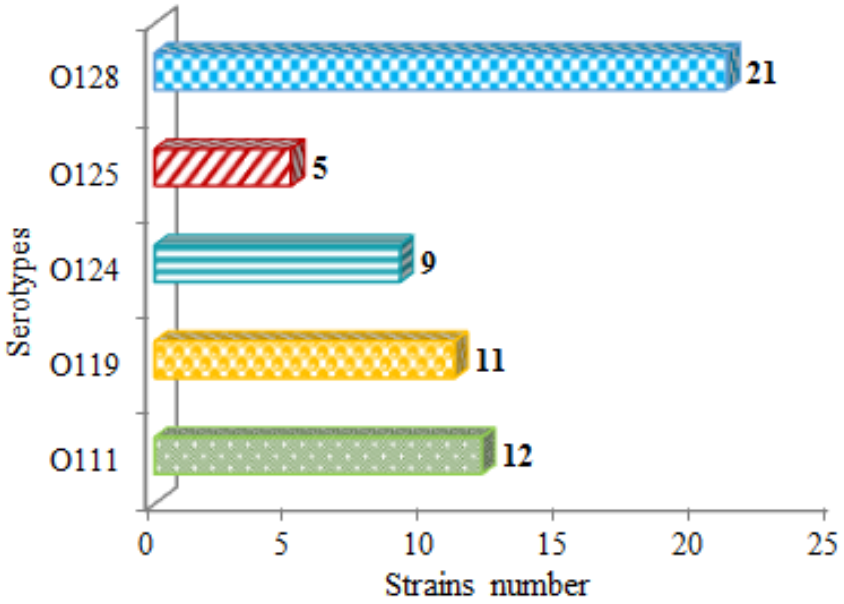

Figure 1. Serotypes of ESBL-producing strains of *Escherichia coli*.

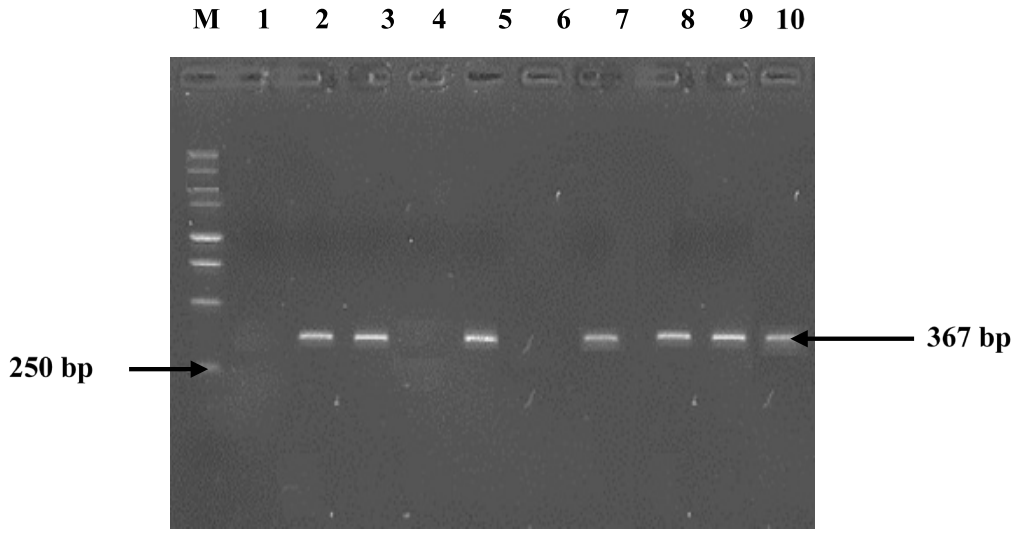

Figure 2. Electrophoretic profile of *eaeA* genes (367 bp).
Note: Line M: Molecular weight marker (BenchTop, 1kb DNA ladder); Line 1: Negative control; Line 2: Positive control strain; Lines 3, 5, 7, 8, 9, 10: Analysed strains hosting *eaeA* gene.

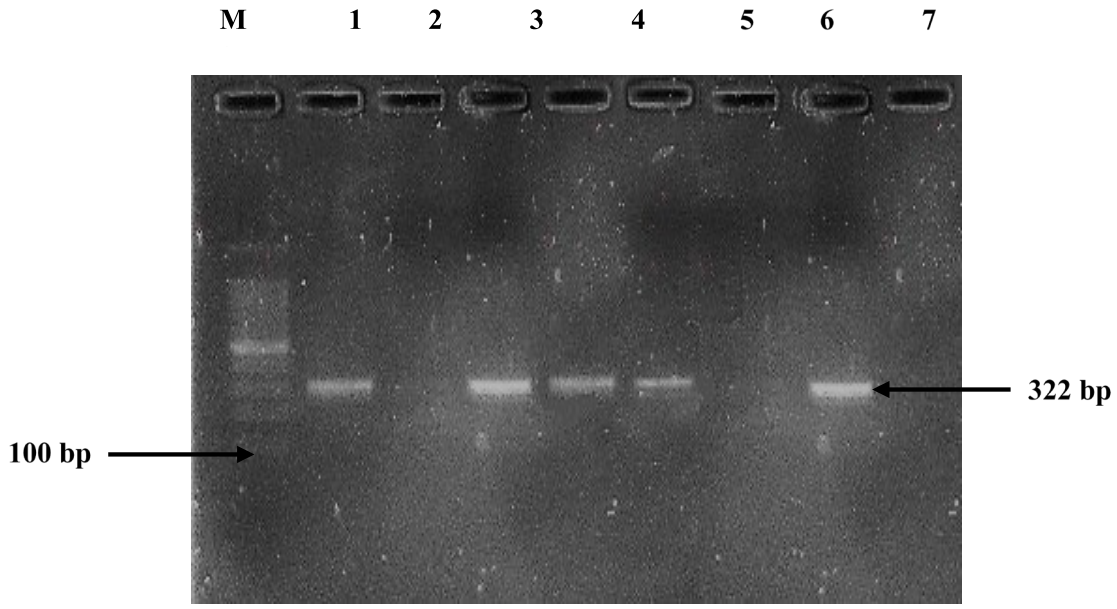

Figure 3. Electrophoretic profile of *eaeA* genes (367 bp).
Note: Line M: Molecular weight marker (BenchTop, 100 bp DNA ladder); Line 1: Positive control strain; Line 2: Negative control; Lines 3, 4, 5, 7: Analyzed strains harboring *lt* genes.

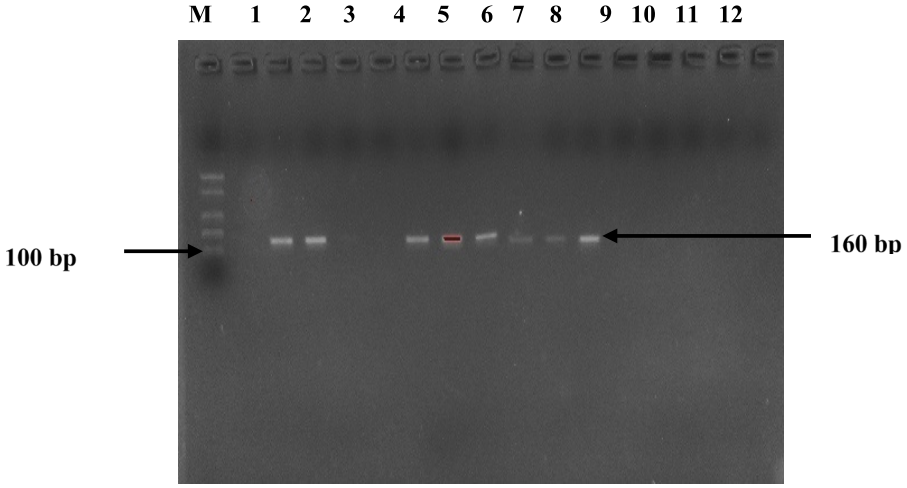

Figure 4. Electrophoretic profile *st* genes (160 bp).
Note: Line M: Molecular weight marker (BenchTop, 100 bp DNA ladder); Line 1: Negative control; Line 2: Positive control strain; Line 3, 6, 7, 8, 9, 10, 11: Strains analysed that host *st* genes.

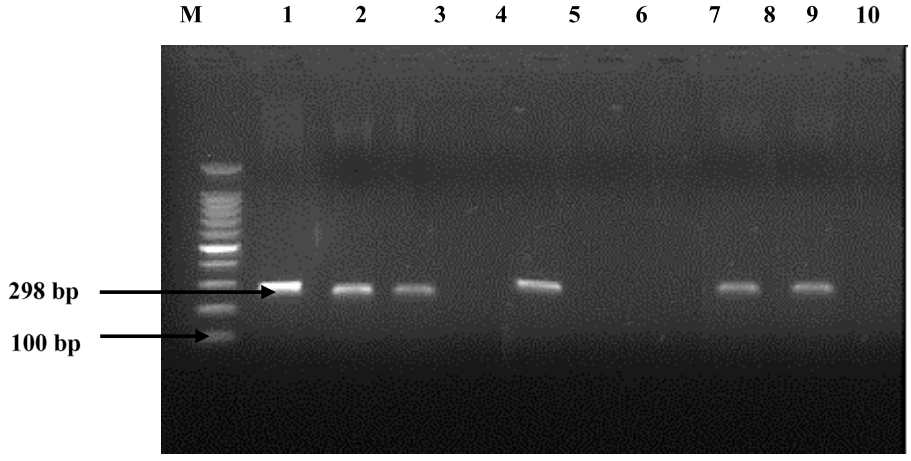

Figure 5. Electrophoretic profile of *Stx2* genes (298 bp).
Note: Line M: Molecular weight marker (BenchTop, 100 bp DNA ladder); Lines 1, 2, 3, 5, 8: Analyzed strains hosting Stx2 genes; Line 9: Positive Control Strain; Line 10: Negative control.

Table 3. Proportions of strains hosting *E. coli* virulence genes in D0 and D4.

	Proportion of strains (%)							
	Proportion of strains (%)		Algo-Bio® treated piglets		Tetracycline treated piglets		Penstrep treated piglets	
Genes	D0	D4	D0	D4	D0	D4	D0	D4
eaeA	0^b	14.2^a	50^a	0^b	75^a	46.6^b	50^a	33.3^b
Stx2	66.6^b	85.7^a	75^a	50^b	50^a	40^b	0^a	0^a
st	33.3^b	71.4^a	75^a	0^b	25^b	33.3^a	50^b	66.6^a
lt	16.6^b	42.8^a	100^a	0^b	75^a	53.3^b	25^a	0^b

Note: D0: day before the beginning of treatment; D4: day at the end of treatment

Table 4. Diameters of inhibition zones for *Escherichia coli.*

			Diameters of inhibition zones (mm)		
	Code	Characteristics	C_1: 100%	C_2: 50%	C_3: 25%
	ATCC 25922	Référence	28.8 ± 1.2	25.5 ± 0.7	20.5 ± 0.7
	EC100	EPEC (*eaeA*)	18.7 ± 0.5	14.6 ± 0.8	10.4 ± 0.5
Escherichia coli	EC72	STEC (*stx2*)	12.5 ± 0.7	10.5 ± 0.8	6 ± 0
	EC96	ETEC (*lt*)	21.5 ± 0.7	19 ± 1.4	16 ± 1.4
	EC05	ETEC (*st*)	26.5 ± 0.7	21.5 ± 0.7	19.5 ± 0.7

Note: Values are mean ± standard deviation of two measurements.

DISCUSSION

The serotyping of *Escherichia coli* strains has been oriented towards the search for strains that are virulent for humans in order to control the risks of propagation of these zoonotic bacteria. ESBL-producing strains of *Escherichia coli* isolated from piglets revealed serogroup O128, O111, O119, O124 and O125. The search for virulence genes in these strains at the beginning and at the end of treatment allowed observing an increase in strains hosting virulence genes *eaeA*, *Stx2*, *st* and *lt* in the control piglets. In general, prevalence's of strains hosting the virulence genes have decreased in piglets treated with Algo-Bio® and tetracycline. On the other hand, a slight increase in proportion of strains carried gene *st* genes was observed in piglets treated with tetracycline from 25% in D0 to 33.3% in D4. In these piglets, proportions of strains carried gene the virulence genes *eaeA*, *Stx2* and *lt* have decreased, but remain relatively high at the end of treatment (D4). The decrease in the levels of strains harboring *eaeA*, *Stx2* and *lt* genes between days D0 and D4 in tetracycline-treated piglets could be explained by the bactericidal action of tetracycline on *Escherichia coli* possessing these genes. The increase in the prevalence of strains harboring *st* virulence genes could be explained by a phenomenon of resistance and transfer of these genes to other *Escherichia coli*. Similar results were observed in piglets treated with Penicillin-Streptomycin combination therapy. Indeed, in these piglets, the proportions of strains harboring *eaeA* and *lt* genes decreased from 50% to 33.3% and 25% to 0 respectively, while the proportions of strains harboring the *st* genes increased from 50% to 66.6%. Tetracycline and Penicillin-Streptomycin did not have a good bactericidal effect on *Escherichia coli* strains harboring *st* virulence genes. This would be explained by the selection pressure exerted by these antibiotics on *Escherichia coli* strains (Kouadio et al., 2017). In control piglets, the significant increase in strains hosting virulence genes between days D0 and D4 could be explained by the lack of drug treatment of these piglets. This led to increase virulent strains, probably due to horizontal transfer of virulence genes. Similar results were observed in the work carried out by (Kouadio et al., 2018). This author showed gene transferability using donor and recipient strains in their work carried out in Côte d'Ivoire on action of amoxicillin on antibiotic resistance in piglet intestinal microbiota strains. *Escherichia coli* strains isolated during this study are not only ESBL-producing but also virulent. Algo-Bio® dietary supplement had good activity in vivo on *Escherichia coli* virulent strains during this study because *eaeA*, *st* and *lt* genes present before treatment were absent at the end of treatment (day D4). In addition, a decrease in the prevalence of strains harboring *Stx2* genes was recorded from 75% (D0) to 50% (D4). This could indicate a bactericidal action of this feed supplement on these strains. (Kouame et al. 2017) evaluated antibacterial activity of Marine Sulphated Polysaccharide (MSP) extract, prepared with green algae, against virulent strains of *Escherichia coli* with significant economic impact in farming systems. In vitro tests carried out by these authors on cell lines have shown the inducing role of several of these molecules on interleukins expression. These marine sulphated polysaccharides would have prebiotic function and would bring health benefit to animal. Prebiotics are functional ingredients that are not digested by animal but act on microbiota by reducing the populations of pathogenic strains. They would favor populations of good bacteria such as Lactobacillus or Bifid bacterium and thus increase animal performance by reducing the risk of disease transmission to humans. Some prebiotics also act by binding pathogenic bacteria, preventing them from colonizing the gastrointestinal tract according to (Lallès et al.,

2007). *eaeA* virulence genes characterize Entero Pathogenic *Escherichia coli* pathovars (EPEC), *Stx2* genes indicate the presence of Shiga Toxin-producing *Escherichia coli* pathovars (STEC), *st* and *lt* genes are specific to EnteroToxin-producing *Escherichia coli* (ETEC). EPEC strains are responsible for diarrhea in humans according to (Livrelli et al., 2007). Shiga-toxin-producing strains of *Escherichia coli* are mainly defined by their ability to produce toxins (stx) called Shiga-like toxin or Shiga-toxins. According to (Nagy et al., 2005), more than 400 different serotypes belonging to STEC pathovar have been isolated from animals but only a small number of serotypes are actually linked to human pathologies such as serotype O111. Strains of ETEC serotype are responsible for diarrhea in humans and colibacillary diarrhea in pigs (Nguyen et al., 2012). The presence of *Escherichia coli* virulent strains in their microbiota is the result of the fragile immunity of weaned piglets (O'Sullivan et al., 2010). However, the presence of these virulent strains in the porcine microbiota constitutes a risk of human contamination. The main mode of transmission of these strains from animals to humans is through of contaminated food consumption. Contamination occurs through consumption of contaminated pork meat or pig products that are in direct or indirect contact with animal faeces and are eaten raw or undercooked such as vegetables. Then, contagion can also occur through contact with animals or their environment and through person-to-person transmission. Efficacy tests of Algo-Bio® carried out in vitro on virulent *Escherichia coli* showed 100%, 50% and 25% activity of the algae extract on certain strains. This could be explained by the antibacterial action of the algae contained in the food supplement. The MSP, the main component of Algo-Bio®, is believed to have a bactericidal action against *Escherichia coli* of porcine origin (Pérès et al., 2001; Ponce et al., 2003). This activity confirms the action of the Algo-Bio® on the carriage of virulent strains of Escherichia coli in weaned piglets (Schmidt. 2010).

CONCLUSION

Algae-based feed supplement (Algo-Bio®) had a good bactericidal action on virulent *Escherichia coli* strains in piglets. Algo-Bio® administration significantly decreased the levels of virulent strains in piglet's intestinal microbiota compared to tetracycline and Penicillin-Streptomycin commonly used in Côte d'Ivoire. However, strains with virulence genes *eaeA*, *Stx2*, *lt* and *st* increased in control piglets. It appears that Algo-Bio® feed supplement could improve piglet's digestive health and promote a better control of the risks of antibiotic resistance in pig farming. So, it could be a good prophylactic alternative to antibiotics in pig farm.

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